Published in final edited form as:

Transplantation. 2008 September 27; 86(6): 854–864. doi:10.1097/TP.0b013e3181861b6c.

Induction of alloanergy in human donor T cells without loss of pathogen or tumor immunity

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Abstract

Background—HLA-mismatched allogeneic hematopoietic stem cell transplantation (HSCT) is limited by acute Graft-versus-Host Disease (aGvHD). Non-selective T cell depletion effectively prevents severe aGvHD but profoundly impairs donor-derived immune reconstitution, increasing infection and disease relapse. The strategy of induction of alloantigen-specific hyporesponsiveness (“alloanergization”) in donor bone marrow by allostimulation with co-stimulatory blockade prior to haploidentical transplantation has demonstrated early promise in reducing severe aGvHD. However, the differential effect of alloanergization on CD4⁺ and CD8⁺ donor T cell subsets and the degree to which beneficial pathogen- and tumor immune responses are retained have not been extensively examined.

Methods—We used an *in vitro* model of alloanergization by allostimulation of human donor T cells with irradiated unrelated recipient peripheral blood mononuclear cells and co-stimulatory blockade with humanized monoclonal anti-B7.1 and -B7.2 antibodies. Residual alloresponses were assessed by proliferation (thymidine uptake, CFSE dye dilution) and cytotoxicity assays. Retention of human herpes virus and tumor-associated antigen-specific immunity was measured with HLA-Class I-restricted pentamers, intracellular cytokine secretion and CD107a assay using 5-color flow cytometry.

Results—Alloanergization of HLA-mismatched donor T cells efficiently and selectively abrogated recipient-specific alloproliferation in both CD4⁺ and CD8⁺ cells while preserving functional CD4⁺ and CD8⁺ immune responses to clinically important human herpes viruses and to the tumor-associated antigen WT1.

Conclusions—Retention of pathogen- and tumor-associated antigen-specific immunity following alloanergization demonstrates that this methodology, which is simple to apply, has potential to improve immune reconstitution whilst limiting alloreactivity after HLA-mismatched HSCT, and deserves additional evaluation in further human clinical trials.

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Author Contributions

JKD designed and performed experiments, analyzed data and wrote the manuscript. DY performed experiments. LMN critically reviewed the manuscript. ECG developed the *ex vivo* cell manipulation and critically reviewed the manuscript.

Declaration of Conflicts of Interest:

The authors have no conflicts of interest to declare.

Keywords

Allogeneic Hematopoietic Stem Cell Transplantation; Donor T cells; Alloanergy; Costimulatory Blockade; Graft-versus-Host Disease; Immune Reconstitution

Introduction

Most of the 30% of individuals requiring allogeneic HSCT who lack a fully HLA-matched donor will have available HLA-mismatched related donors.(1) However, donor-recipient HLA-mismatches increase incidence and severity of aGvHD and treatment failure.(2) AGvHD, mediated predominantly by recipient alloantigen-specific donor T cells which expand *in vivo* post-HSCT, may be efficiently prevented by non-selective T-cell depletion (nsTCD), even in haploidentical HSCT.(3,4) However, nsTCD removes pathogen- and tumor-specific donor T cells, delays immune reconstitution, increases infectious complications, and may increase relapse rates.(5–7)

Various strategies of selective allodepletion (SAD) have been developed to remove alloreactive T cells from donor grafts to prevent aGvHD while preserving pathogen- and tumor-specific immunity. Most approaches use a common platform of *ex vivo* donor T cell allostimulation after which alloreactive donor T cells, identified by activation markers, metabolic activity or proliferation are removed or destroyed.(8–11) Alternatively, alloanergy can be induced in donor T cells prior to HSCT. Human T cells require at least two signals from antigen-presenting cells (APCs) to become activated: T cell receptor ligation by cognate antigen (Signal 1) and positive costimulatory signals (Signal 2).(12) The CD28 receptor delivers the predominant Signal 2 to human T cells. This signal may be blocked by monoclonal antibodies binding to the CD28 ligands, CD80 (B7.1) and CD86 (B7.2) on APCs. T cells receiving Signal 1 without Signal 2 enter a state of antigen-specific hyporesponsiveness (anergy), failing to proliferate after restimulation with original antigen, even when positive costimulatory signals are present. (13) Alloanergy can thus be induced in donor T cells by *ex vivo* stimulation with recipient APCs in the presence of costimulatory blockade (CSB).(14) This technique has several advantages over existing SAD approaches. The *ex vivo* culture period is short, requiring no further cell processing, sorting or exposure to hazardous agents. Furthermore the technique does not utilize activation marker expression, which may vary between donor-recipient pairs, nor is it limited to subsets of alloreactive T cells (e.g. only those that proliferate after allostimulation).

In an early clinical study of transplantation of alloanergized bone marrow (BM), using the fusion protein CTLA4-Ig to block CD28-mediated co-stimulation, BM containing large doses of haploidentical donor T cells were transplanted without excess severe aGvHD.(15) However, practical constraints and limitations of immunological assays to assess antigen-specific immunity available at the time prevented us from directly assessing the beneficial immunity provided by alloanergized donor T cells *in vivo*.

We have now developed a strategy to alloanergize donor peripheral blood mononuclear cells using humanized anti-B7.1 and B7.2 antibodies, which bind to B7.1 and B7.2 with similar avidity and kinetics, in contrast to “first generation” CTLA4-Ig which has inferior B7.2 binding kinetics.(16) In this report, we use an *in vitro* HLA-mismatched model to examine the reduction of alloresponses and the retention of beneficial immune responses after alloanergization using humanized anti-B7.1 and B7.2 antibodies prior to implementation of this strategy in a new clinical study.

We show directly for the first time that this strategy abrogates alloproliferation in CD8⁺ as well as CD4⁺ cells. Furthermore, the strategy preserves functional CD4⁺ and CD8⁺ immune responses to human herpes viruses as well as to the tumor-associated antigen (TAA) WT1, which could provide a valuable Graft-versus-Leukemia (GvL) effect. These data confirm that the technique of alloanergization, already demonstrated to have promise in ameliorating severe aGvHD in a clinical setting, and significantly easier to implement than many existing SAD approaches, does not adversely affect beneficial donor-derived immune responses. The technique is thus a viable alternative to established SAD strategies for provision of non-alloreactive donor T cells to improve immune reconstitution after HLA-mismatched HSCT.

Material and Methods

Alloanergization of donor T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh leucocyte filters from volunteer blood donors by Ficoll-Paque density gradient centrifugation (Amersham Biosciences, Piscataway, NJ) on an institutional review board-approved protocol. To generate alloanergized responders, 10⁷ responder PBMCs and γ -irradiated (3.5Gy) stimulator PBMCs from unrelated volunteer donors were co-cultured for 72 hours in 25cm² flasks (Corning, NY) in 20mL complete media (CM; RPMI, penicillin/streptomycin, 10% human AB serum, Sigma-Aldrich (SA), St. Louis, MO) with 100 μ g humanized monoclonal anti-B7.1 and anti-B7.2 antibodies (Wyeth, Madison NJ), washed and resuspended. 10⁷ responders alone were set up in parallel culture without allostimulation ("Untreated responders").

Thymidine incorporation assay

10⁵ untreated or alloanergized responders were cultured with equal numbers of γ -irradiated stimulator PBMCs or CD3 and CD28 antibodies (Beckman Coulter (BC), Fullerton, CA) in 200 μ L CM in 96-well plates (Nunc, Rochester, NY) for 5 days. 0.037MBq (1 μ Ci) ³H-thymidine/well (Amersham) was added 18 hours before harvesting with a Tomtec automated cell harvester (Wallac, Hamden, CT) and thymidine incorporation measured by Microbeta liquid scintillation counting (Perkin Elmer, Meriden, CT). Results were standardized by calculating "percentage residual alloproliferation" as 100*counts per minute (cpm) (alloanergized responders + allogeneic stimulators) – cpm (alloanergized responders + autologous stimulators) $\div$ cpm (untreated responders + allogeneic stimulators) – cpm (untreated responders + autologous stimulators).

CFSE proliferation assay

10⁷ untreated or alloanergized responders were labeled with 10 μ M Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE, Invitrogen, Carlsbad, CA) in 500 μ L phosphate-buffered saline for 8 minutes at room temperature (RT). 10⁷ CFSE-labeled responders and γ -irradiated allogeneic or autologous stimulators were co-cultured in 20 mL CM in 25cm² flasks, then stained with Phycoerythrin (PE)-Texas Red (ECD)-, PE-Cyanine(Cy)5- and PE-Cy7-conjugated monoclonal antibodies to CD3 (clone UCHT1), CD4 (13B8.2) and CD8 (SFC121Thy2D3) (all BC). Responders were identified by forward-and side-scatter and percentage proliferating CD4⁺ and CD8⁺ cells determined by CFSE dilution.(17) Percentage alloproliferative cells was determined as { % proliferating (allogeneic stimulators) – % proliferating (autologous stimulators)}.

Calculation of alloproliferative precursor frequencies

Between 5 and 8 individual peaks (representing cell divisions) could be resolved in untreated CFSE-labeled responders after allogeneic stimulation. However the percentage of alloanergized responders proliferating after allogeneic restimulation was too low for accurate

resolution of individual peaks required for calculation of allospecific precursor frequency (alloPF) by the method described by Lyons, so the model approximating dividing cells to a single peak described by Martins was employed thus: $\text{alloPF} (\%) = 100c/(2^n + c) - (2^n * c)$ where $c = (\text{number CFSE}^{\text{dim/neg}} \text{ responders} / \text{number CFSE}^{\text{dim/neg}} \text{ responders} + \text{number CFSE}^{\text{bright}} \text{ responders})$ following alloantigen stimulation, $n = \log_2[b/a]$ where $a = \text{Median fluorescence Intensity (MFI; CFSE}^{\text{dim/neg}} \text{ responders)}$ and $b = \text{MFI (CFSE}^{\text{bright}} \text{ responders)}$.(17,18) Values after autologous stimulation were subtracted from those after allogeneic stimulation.

Generation of allospecific effectors and measurement of allospecific cytotoxicity

Allospecific effectors were generated by culture of 10^7 untreated or alloanergized responders and γ -irradiated stimulators in 20mL CM in 25cm² flasks for 7 days. A flow cytometric apoptosis assay was used for determination of cytotoxic activity.(19) 5×10^6 cultured target PBMCs were labeled with the fluorescent dye PKH26 (SA) at RT for 5 minutes and resuspended at $10^5/\text{mL}$ in CM and added to allospecific effectors in V-bottom 96-well plates (Corning) to a volume of 200 $\mu\text{L}/\text{well}$, incubated for 4 hours then stained with Annexin V-FITC for 15 minutes in Annexin V staining buffer (BD). A minimum of 10^4 PKH26⁺ targets were acquired. Spontaneous (background) target cell apoptosis was assessed in target cells incubated alone. Allospecific cytotoxicity (AC) was calculated as: $\text{AC} = 100 * (\% \text{ Annexin V}^+ \text{ targets (test sample)} - \% \text{ Annexin V}^+ \text{ targets alone}) / 100 - \% \text{ Annexin V}^+ \text{ targets alone}$. Immunomagnetic beads (Miltenyi, Auburn, CA) were used to deplete CD56⁺ cells from allospecific effectors according to the manufacturer's instructions. CD56⁺ cells were reduced 100-fold (to less than 0.1%).

MHC Class I-restricted pentamers

HLA-A2⁺ donors were identified by staining PBMCs with HLA-A2 fluorescein-isothiocyanate (FITC)-conjugated antibody (BB7.2, BD). 10^6 responders from HLA-A2⁺ donors were stained for 15 minutes at RT with PE-conjugated HLA-A0201-CMV pp65 (NLVPMVATV) or HLA-A0201-EBV BMLF-1 (GLCTLVAML) pentamers, titrated for optimal staining (Proimmune, Oxford) and then with CD3-FITC and CD8-PE-CY5 (BC), and 5×10^4 CD3⁺CD8⁺ events acquired. PBMCs from HLA-A2⁻ donors were used to assess non-specific pentamer binding. Frequencies of pentamer⁺ cells were expressed as percentage of CD3⁺CD8⁺ cells, above that seen in HLA-A2⁻ controls.

Measurement of pathogen- and tumor-associated antigen-specific immune responses

Intracellular cytokine secretion flow cytometry (ICC) and CD107a assays were performed as described.(20,21) 10^6 alloanergized or untreated responders were suspended 0.5mL CM in polypropylene tubes (BD) with 2 μg anti-CD28 (Clone CD28.2, Immunotech, Fullerton, CA) and stimulated with no antigen, mock-, VZV-, HSV- and CMV-infected human foreskin fibroblast cell lysates (Colorado Health Sciences, Denver CO) or 110 μg of overlapping 15-mer peptides from the WT1 protein (WT1 pepmix; JPT, Berlin) or 5 μg Staphylococcal Enterotoxin B (SEB, SA) for 12 hours. 5 μg Brefeldin A (SA) was added after 4 hours. For CD107a assay, 20 μL anti-CD107a FITC (H4A3, BD) was added to cells prior to stimulation. After stimulation, cells were washed and stained with CD3-ECD, CD4-PE-Cy5, CD8-PE-Cy7 and CD107a-FITC (later with CD107a-PECy5 (BD), which had superior staining characteristics in conjunction with CD4-PE, CD3-ECD and CD8-PE-Cy7). For ICC, cells were fixed, permeabilized and stained with PE-(later FITC-) conjugated IFN- γ (4SB3), IL2 (MQ1-17H12) and/or IL10 (JES3-19F1) antibodies (BD) for 30 minutes at 4°C. A minimum of 4×10^4 CD4⁺ and CD8⁺ events were acquired. The summed frequency of IFN- γ ⁺ and IL2⁺ cells was used to assess CD4⁺ immune responses to human herpes viruses as this polyfunctional pattern of Th1 cytokine secretion best represents combined central and effector human memory CD4⁺ responses to persistent viral antigens.(22) Intra-assay co-efficient of

variation (CV) was calculated according to the Poisson distribution as $CV=100/\sqrt{n}$ (n = number of positive events). 95% confidence intervals (CI) for frequency of positive events were calculated as $1.96 \times (CV \times \text{mean test frequency})$. The percentage retention of antigen-specific cells was calculated as $100 \times (\text{frequency of alloanergized antigen-specific cells}) / \text{frequency of untreated antigen-specific cells}$. For CMV-specific proliferation assays, 10^6 responders from CMV-seropositive donors were CFSE-labeled and stimulated with 125 μ g mock- or CMV-infected lysate in 25cm² flasks for 7 days. Cells were washed, stained with CD3-ECD, CD4-PE-Cy5, CD8-PE-Cy7 antibodies and events acquired. The percentage of CMV-specific proliferating cells was determined as % proliferating responders (CMV lysate) - % proliferating responders (mock lysate).

Statistical considerations

Statistical analysis was performed with Graph Pad Prism (San Diego, CA). A p value of <0.05 was used to reject the Null Hypothesis.

Results

Alloanergization efficiently and specifically reduces alloproliferation in CD4⁺ and CD8⁺ cells

We used thymidine incorporation to measure proliferation of untreated and alloanergized responders in 12 unrelated stimulator-responder pairs after 5 days of allo(re)stimulation. Alloproliferation was significantly lower in alloanergized responders than untreated responders. In contrast, proliferation after mitogenic stimulation in alloanergized responders was not significantly reduced when compared to untreated responders. Median residual alloproliferation in alloanergized responders was 1.2% (range 0–7.3%) whereas median residual proliferation to mitogens was 75% (37–205), representing a median 85-fold reduction (range 14-> 1000-fold) in alloproliferation but only a 1.3-fold reduction (0.5–2.7) in mitogen-specific proliferation after alloanergization (Figure 1).

We then used CFSE-dye dilution to quantify proliferation of CD4⁺ and CD8⁺ responders after allo(re)stimulation in a further 12 unrelated stimulator-responder pairs. Alloanergization resulted in a median 50-fold reduction and 7-fold reduction in the percentage of CD4⁺ and CD8⁺ cells proliferating after allostimulation when compared to untreated responders (Figure 2A, upper panels). In contrast, there was no difference in proliferating CD4⁺ or CD8⁺ cells after third-party allostimulation in first-party alloanergized and untreated responders, indicating that the alloanergization process was specific to the allostimulators used during anergy induction (Figure 2A, lower panels). We assessed alloproliferation after 14 days of allorestimulation in 4 of these stimulator-responder pairs. The reduction in first party alloproliferation (with preservation of third party responses) was maintained in all pairs tested in both CD4⁺ and CD8⁺ cells, indicating alloanergization abrogated rather than delayed subsequent alloresponses (Figure 2B). In common with many other groups, we found that the addition of IL2 (100iu/ml) to alloanergized responders during allorestimulation restored their proliferative capacity (data not shown).

We also calculated the allospecific precursor frequency using CFSE dye dilution in untreated and alloanergized responders after 7 days of allo(re)stimulation to assess what proportion of allospecific responder cells failed to divide after alloanergization. The median alloPF was 0.9% (range 0.2–2.3%) in untreated CD4⁺ responders, somewhat lower than that reported by Martins (who employed pooled donor allostimulators) but consistent with that reported by Godfrey (1.1%), who in common with our approach used single donor allostimulators.(11,18) The median alloPF in untreated CD8⁺ responders was 0.6% (0.16–3). We observed a median 86-fold and 20-fold reduction in CD4⁺ and CD8⁺ alloPF respectively in alloanergized responders,

indicating that 99% of allospecific CD4⁺ and 95% of allospecific CD8⁺ cells fail to proliferate after alloanergization.

Alloanergization limits generation of allospecific effector cells

We then asked whether alloanergization was sufficient to prevent the generation of allospecific effectors. We generated first and third party allospecific effector cells from both first party-alloanergized and untreated responders and assessed the efficacy and specificity of effectors at inducing apoptosis of allogeneic targets. First party allogeneic target apoptosis was reduced 3–5 fold with allospecific effectors generated from first party-alloanergized responders compared to untreated responders at all E:T ratios tested whilst third party allogeneic target apoptosis was retained using allospecific effectors generated from first party-alloanergized responders (Figure 2C left and middle panels). To determine the contribution of alloreactive NK cells to residual target cell apoptosis seen with allospecific effectors generated from alloanergized responders, we depleted effector cells of CD56⁺ cells immediately prior to allospecificity assays. Reduced first party target cell apoptosis was seen after CD56-depletion of allospecific effectors from both untreated and alloanergized responders (Figure 2C right panel) with residual first party target cell apoptosis only detectable at an E:T ratio of 20:1.

Alloanergization does not impair functional CD4⁺ responses to viral pathogens

In order to determine the impact of alloanergization on virus-specific T cell response, we first investigated virus-specific Th1 cytokine responses using ICC.(23) We were able to reproducibly detect virus-specific Th1 cytokine⁺ CD4⁺ cells at frequencies as low as 0.1% (95% CI $\pm$ 0.01%) of CD4⁺ cells. The frequency of positive events in negative controls was consistently <0.2%. Responses to viral lysates were considered positive if (test sample - 95% CI) was greater than (negative control + 95% CI) and the absolute frequency for (test sample - negative control) was greater than or equal to 0.1%. PBMCs from healthy donors were screened for virus-specific Th1 cytokine⁺ CD4⁺ cells by ICC after 72 hours in culture. Viability after culture of responders was >95% by PI exclusion. Donors were identified with detectable VZV, HSV or CMV-specific CD4⁺ cells with frequencies consistent with published reports for healthy donors (Figure 3A).(24) The frequency of virus-specific Th1 cytokine⁺ CD4⁺ cells was not significantly different in untreated and alloanergized responder cells from these donors, Table 1. The median percentage retention of virus-specific Th1 cytokine⁺ CD4⁺ cells was 117% (range 42–235) for VZV, 80% (51–178) for HSV and 97% (49–162) for CMV.

Since reduction in Th1/Th2 ratios of CMV-specific CD4⁺ cells has been associated with impaired CMV-specific immunity after solid organ transplantation, we next determined the Th1/Th2 ratio of CMV-specific CD4⁺ cells following CMV-lysate stimulation in 3 CMV-seropositive healthy donors (25). There was no significant difference in Th1/Th2 ratio of CMV-specific CD4⁺ cells in untreated and alloanergized responders (median 18 (range 7–24) vs. 16 (range 9–18, Figure 3B).

In view of the profound impairment of allospecific proliferation seen after alloanergization, it was also necessary to determine whether alloanergization affected the proliferative capacity of virus-specific CD4⁺ cells. Proliferative responses in CD4⁺ cells from 5 CMV-seropositive healthy donors stimulated with CMV lysate were retained after alloanergization in all donors tested (Figure 3C). The median retention of CMV-specific CD4⁺ proliferation was 89% (range 62–128%).

Functional virus-specific CD8⁺ responses are also preserved after alloanergization

In view of the novel finding that alloanergization reduced allospecific proliferation in CD8⁺ cells, the impact of alloanergization on quantitative and functional CD8⁺ cell immunity to pathogens was assessed. Healthy HLA-A2⁺ donors were screened for CMV- and EBV-specific

T cells with HLA A0201-NLVPMVATV (CMV) and -GLCTLVAML (EBV) pentamers. Frequencies of CMV- and EBV-pentamer-specific cells were similar in untreated and alloanergized responders (median 0.67% (range 0.57–3.1) vs. 0.88% (0.27–2.92), and 0.21% (0.12–0.31) vs. 0.20 (0.11–0.48) respectively). The median retention of pentamer⁺ cells was 101% (range 40–149) (CMV) and 105% (65–110) (EBV), (Figure 4A).

Functional CD8⁺ responses after viral lysate stimulation were assessed by CD107a upregulation, a flow cytometric assay that correlates directly with pathogen- and tumor cell-specific cytotoxicity assays.(21,26) Criteria for response were identical to those for detection of virus-specific Th1 CD4⁺ cells by ICC. CD107a⁺ CD8⁺ responses in healthy donors correlated significantly with the presence of detectable virus-specific Th1 cytokine⁺ CD4⁺ cells (data not shown).

The frequency of virus-specific CD107a⁺ CD8⁺ cells was not significantly different in untreated and alloanergized responders Figure 4B and Table 1. The median retention was 80% (range 78–123) for VZV, 102% (77–150) for HSV and 83% (75–310) for CMV.

Tumor-associated antigen-specific CD8⁺ and CD4⁺ responses are retained after alloanergization

In order to assess retention of potential GvL activity after alloanergization, we measured immune responses after stimulation with a pepmix derived from WT1. Five of 11 healthy donors (2 HLA-A2⁺, 3 HLA-A2[−]) screened had detectable WT1 pepmix-specific IFN-γ⁺ CD8⁺ cells, and 4 had detectable WT1 pepmix-specific CD107a⁺ CD8⁺ cells. WT1 pepmix-specific IFN-γ⁺ CD4⁺ cells were also detectable in 5 donors. Three such donors also had detectable frequencies of both WT1 pepmix-specific IFN-γ⁺ and WT1 pepmix-specific CD107a⁺ CD8⁺ cells, Table 2. WT1 pepmix-specific IFN-γ⁺ CD8⁺ cells were retained after alloanergization in 4 of 5 donors with a median retention of 80% (range 0–125) and WT1 pepmix-specific CD107a⁺ CD8⁺ cells were retained after alloanergization in all donors with a median retention of 97% (range 69–114, Table 2). Importantly, frequencies of WT1 pepmix-specific IFN-γ⁺ CD4⁺ cells in alloanergized responders were also retained in 4 of 5 donors with a median retention of 111% (range 0–181, Table 2).

Discussion

Although early clinical experience suggested alloanergized haploidentical BMT resulted in lower incidences of severe aGvHD than historical reports of T cell replete haploidentical HSCT, and fewer viral infections than nsTCD haploidentical HSCT, the provision of beneficial immunity by alloanergized donor T cells were not directly addressed.(27–30) Using *in vitro* models we now show alloanergization of donor T cells effectively reduces alloresponses in both CD4⁺ and CD8⁺ cells, without reducing pathogen- and TAA-specific immune responses.

Although the relative benefits of the multitude of different approaches developed to selectively reduce alloreactivity cannot be accurately determined without direct *in vitro* and *in vivo* comparisons, the reduction in alloresponses we observed *in vitro* were similar to those reported with other SAD strategies. Following alloanergization with humanized anti-B7.1/2 antibodies, residual alloreactivity was 1.2% (measured by thymidine incorporation), 3-fold less than that reported using CTLA4-Ig +/- cyclosporine, 3–8-fold less than SAD based on activation marker expression using PBMCs as APCs,(8,9,14,31) and comparable with CD25-immunotoxin-mediated SAD using lymphoblastoid cell lines as APCs.(8) The reduction of alloreactive CD8⁺ precursor frequency after alloanergization was comparable to SAD by photodynamic purging.(32) Our strategy reduced alloresponses more efficiently in CD4⁺ than in CD8⁺ cells, probably reflecting the higher frequency of human CD28[−]CD8⁺ (30%) compared with CD28[−]CD4⁺ cells (5%) found in healthy donors. Interestingly, CD8⁺ cells proliferating after

alloanergization were enriched with CD28⁻ cells (data not shown). Such cells may receive costimulation via alternative receptors (e.g. from CD40L or ICOS), which could serve as additional targets of CSB to further improve alloanergization.(23)

The reduction in residual allocytotoxicity we observed with alloanergized responders after CD56 depletion supports the theory that alloreactive NK cells (alloNK), which are not affected by CD28-mediated CSB, contribute to residual allocytotoxicity after alloanergization.(33)

AlloNK aid engraftment without inducing GvHD in murine models, and may provide beneficial GvL activity in human haploidentical HSCT, particularly in the absence of alloreactive T cells,(34,35). Significantly, AlloNK upregulate CD25 and CD69 upon activation and would therefore be removed by alternate activation-marker-based SAD strategies.(8,36)

Our data confirm prior observations of retention of EBV- and CMV-specific CD8⁺ responses reported after a somewhat different alloanergization strategy.(31) Importantly, we have shown for the first time that virus-specific Th1 responses and CMV-specific proliferation are retained in CD4⁺ cells after alloanergization. The retention of pathogen-specific responses in CD4⁺ cells has great significance in light of recent data demonstrating that effective provision of pathogen-specific immunity post-HSCT requires antigen-specific memory CD4⁺ T cells as well as CD8⁺ effector T cells.(37–39) Furthermore, provision of protective immunity against CMV post-HSCT has been shown to correlate with CMV-specific proliferative responses.(40) Alloanergization by blockade of B7/CD28 pathway has been previously shown to result in the generation of alternately-activated macrophages which had reduced capacity to stimulate autologous PPD-specific T cell responses in healthy donors. Importantly, the retention of CD4⁺ responses specific to virally-infected cell lysates demonstrated that antigen processing and presentation of these antigens were not significantly impeded by our strategy of alloanergization. (41)

Alloanergization may target alloreactive cells more specifically than alternative techniques of SAD. It is likely that the CMV-specific T cells spontaneously secreting IFN- γ ⁺ present in many CMV-seropositive healthy donors would be removed by SAD strategies utilizing increased metabolic activity to identify alloreactive cells.(42) Indeed, CD69-mediated SAD has been shown to remove CD69^{weak} CMV-specific CD8⁺ cells.(43) In contrast, human CD8⁺CD28⁻ T cells, which have been shown to contain greatly expanded functional CMV-specific memory clones, would not be affected by CD28 CSB-mediated alloanergization.(44)

The retention of functional WT1 pepmix-specific T cells after alloanergization may provide one immunological mechanism whereby a GvL effect could be retained after alloanergization. WT1 is overexpressed on acute and chronic leukemic blasts and many solid tumor cells. In contrast to other TAA-specific T cell responses, WT1-specific T cells are detectable at low frequency in more than 50% of healthy individuals and also in leukemia patients.(45) Furthermore such cells are found at increased frequency after allogeneic HSCT in patients with myeloid leukemia, and donor-derived WT1 peptide-specific IFN- γ ⁺ CD8⁺ cells correlated with disease remission after allogeneic HSCT in HLA A*0201⁺ patients with acute lymphoblastic leukemia, suggesting such cells may mediate a clinically significant GvL effect.(46,47) The potential for alloanergized cells to exert a significant GvL effect is supported by the retention of both WT1 pepmix-specific IFN- γ ⁺ CD8⁺ and CD4⁺ cells and WT1 pepmix-specific CD107a⁺ CD8⁺ cells indicative of lytic effector function. Furthermore, by using a large pool of WT1-derived peptides to stimulate functional T cell responses, we have been able to demonstrate the retention of tumor-associated antigen-specific immune responses in both HLA A2⁺ and non-HLA A2⁺ individuals. This may have more closely represented the repertoire of WT1-derived antigens presented by residual leukemia cells after allogeneic HSCT than responses related to a single HLA-restricted peptide. Although It would be of interest to

investigate the retention of T cell responses specific for additional TAAs overexpressed in hematological malignancies (PRAME, survivin), T cell responses specific to such TAA are found only in a minority of healthy donors,(48,49) suggesting limited potential for a widely applicable donor-derived GvL effect after allogeneic HSCT.

There are considerable practical advantages of generating alloanergized donor T cells for adoptive use after allogeneic HSCT. More than 10^8 alloanergized donor T cells can routinely be generated from 200mL of blood, sufficient for an adoptive T cell dose of 10^6 /kg for a 70kg recipient, ten-fold more than the dose of selectively allodepleted T cells reported to improve immune reconstitution without significant aGvHD after haploidentical HSCT in recent key clinical studies.(32,50) Unlike lymphoblastoid cell lines used as recipient APCs in one such study,(8) PBMCs are obtained easily and require no *ex vivo* culture prior to use. Possible induction of anergy to tumor-associated antigens by tumor cells contaminating recipient PBMCs could be avoided by using as allostimulators PBMCs from a family member sharing the recipient haplotype not shared by the donor and recipient. Exposure of alloanergized donor T cells to high levels of cytokines, which has been shown to result in temporary reversal of alloanergy *in vitro*, could be minimized by delaying infusion until several weeks after HSCT, when cytokines induced by conditioning chemo-radiotherapy have subsided.(23)

Alloanergization has potential for generation of non-alloreactive T cells for adoptive use to reduce treatment failure after HLA-mismatched HSCT, and warrants investigation in further clinical studies. A clinical trial of adoptive alloanergized donor T cell infusion after haploidentical HSCT is currently underway to determine the optimal dose of such T cells able to improve immune reconstitution without causing severe aGvHD.

Acknowledgements

Funding Sources

This research was funded in part by the National Institutes of Health grant U19 CA100625.

JKD is a recipient of a Career Development Award from the Leukemia and Lymphoma Society.

Additional Acknowledgements

The authors would like to thank Dr Laurence Cooper for helpful comments on the manuscript.

Abbreviations

AC, Allogeneic toxicity
aGvHD, acute Graft-versus-Host Disease
alloPF, allo-Precursor Frequency
APC, Antigen Presenting Cell
BMT, Bone Marrow Transplantation
CFSE, Carboxyfluorescein Diacetate Succinimidyl Ester
CI, Confidence Interval
CM, Complete Media
CPM, Counts per Minute
CSB, Costimulatory Blockade
CTLA4-Ig, Cytotoxic T Lymphocyte Antigen 4 Immunoglobulin
CV, Co-efficient of Variation
Cy, Cyanin
FITC, Fluorescein-isothiocyanate
GvL, Graft-versus-Leukemia
HSCT, Hematopoietic Stem Cell Transplantation

ICC, Intracellular Cytokine Cytometry
 ICOS, Inducible T cell Costimulator
 MFI, median Fluorescence Intensity
 nsTCD, non-selective T Cell Depletion
 PE, Phycoerythrin
 PI, Propidium Iodide
 SAD, Selective Alodepletion
 SEB, Staphylococcal Enterotoxin B
 TAA, Tumor-associated Antigen
 WMPT, Wilcoxon matched Pair Test

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Figure 1. Alloanergization consistently and efficiently reduces alloproliferation without reducing proliferative responses to mitogenic stimulation

1A–D Scatter plots of proliferation measured by thymidine incorporation of untreated and alloanergized responders in 12 unrelated stimulator-responder pairs after 5 days of allo(re) stimulation are shown. Horizontal bars represent median values and p values are for 2 tailed Wilcoxon Matched Pair Tests. Alloproliferation was significantly reduced in alloanergized responders compared to untreated responders (median 204 cpm, range 0–1790 vs. 11696 cpm, range 7814–59260 Figure **1A**). In contrast, there was no significant reduction in proliferation after mitogenic stimulation in alloanergized responders (6331 cpm, range 1735–19584) when compared to untreated responders (median 11296 cpm, range 844–28592, **1B**) Median residual alloproliferation in alloanergized responders was 1.2% (range 0–7.3%) whereas median residual proliferation to mitogens was 75% (37–205, **1C**) representing a median 85-fold reduction (range 14–>1000-fold) in alloproliferation but only a 1.3-fold reduction (0.5–2.7) in mitogen-specific proliferation after alloanergization (**1D**).

Figure 2. Alloantigenization significantly and specifically reduces alloproliferation of both CD4⁺ and CD8⁺ responders and the generation of functional allospecific effectors

Proliferating cells were quantified by CFSE-dilution after allo(re)stimulation of untreated or first party alloantigenized responders in a further 12 unrelated stimulator-responder pairs. The percentage of first-party alloantigenized CD4⁺ responders proliferating after first-party allo(re) stimulation was consistently and significantly lower than that of untreated CD4⁺ responders (median 0.5% (range 0–7.4%) vs 28% (7.3–87), a median 50-fold reduction). The percentage of alloantigenized CD8⁺ responders proliferating after allo(re)stimulation was also markedly lower than that of untreated CD8⁺ responders (median 4.8% (0–15) vs 26% (5–79), a median 7-fold reduction, (Figure 2A upper panels). In contrast, there was no significant reduction in the percentages of responders proliferating after third-party allostimulation in first-party alloantigenized cells in both CD4⁺ and CD8⁺ cell subsets. (Figure 2A lower panels). Different colors are used to identify individual pairs, horizontal bars represent median values. P values are for 2-tailed Wilcoxon Matched Pair Tests (WMPT) comparing untreated and alloantigenized

responders. Alloresponses were also assessed after 14 days of allostimulation in 4 of these 12 pairs. The reduction in first party alloproliferation (Figure 2B, upper panel) with preservation of third party responses (Figure 2B, lower panel), was maintained in all pairs tested in both CD4⁺ and CD8⁺ cells, indicating alloenergization abrogated (rather than delayed) subsequent alloresponses.

Apoptosis of first party targets was 3–5-fold lower with allospecific effectors generated from first party alloenergized responders than with allospecific effectors generated from untreated responders whereas equivalent apoptosis of third party targets was seen. (Figure 2C, left and middle panels) Mean (+/–standard deviation) of three separate experiments (each performed in triplicate) is shown. Residual Apoptosis of first party targets was reduced after CD56-depletion of alloenergized allospecific effectors. Mean (+/–standard deviation) of three further separate experiments (each performed in triplicate) is shown in Figure 2C, right panel (using different stimulator-responder pairs to those shown in the left panel). E: T, Effector: Target

Figure 3. Functional pathogen-specific CD4⁺ responses are retained after allogeneization

3A, Viral lysate-specific CD4⁺ Th1 cytokine⁺ cells were detectable by intracellular cytokine flow cytometry (ICC) in both untreated and allogeneized responders. Dot plots from a representative donor (A) are shown. Cells were passed through CD3⁺ and CD4⁺ gates. Numbers in boxes represent percentages of positive events in CD4⁺ cells.

3B, Allogeneization did not affect the Th1/Th2 ratio (IFN-γ/IL10) of CMV-specific CD4⁺ cells after stimulation with CMV lysate in three separate experiments with CMV-seropositive donors (representative dot plots from one experiment are shown).

3C, CMV-specific proliferative responses (assessed by CFSE dye dilution), shown for 5 donors (*U*, *Y*, *AA*, *AE* and *AH*) were maintained in all donors tested after alloanergization. Mock, mock infected cell lysate; SEB, staphylococcal enterotoxin B.

Figure 4. Pathogen-specific CD8⁺ cells are retained after alloanergization

4A, Frequencies of CMV-specific HLA A*0201 NLVPMVATV pentamer⁺ cells and EBV-specific HLA A*0201 GLCTLVAML pentamer⁺ cells, shown for 8 HLA-A2⁺ donors (AE, AN, AB, AY, AH, AD and AG), were equivalent in untreated and alloanergized responders. Pentamer⁺ frequencies are expressed as percentage of CD3⁺CD8⁺ cells.

4B, Functional CD8⁺ responses were measured by upregulation of the lysosomal-associated protein CD107a after stimulation with viral lysates. Dot plots from one representative donor are shown. Cells were passed through CD3⁺ and CD8⁺ gates. Numbers in boxes represent percentages of positive events in CD8⁺ cells.

Table 1
Frequencies of human herpes virus-specific cells in untreated and alloantigenized donor T cells

Donor	Th1 cytokine ⁺ cells, percentage of CD4 ⁺ cells						CD107a ⁺ cells, percentage of CD8 ⁺ cells					
	VZV- specific			HSV- specific			VZV- specific			HSV- specific		
	Untreated	Anergized		Untreated	Anergized		Untreated	Anergized		Untreated	Anergized	
A	0.81	0.34		0.96	0.49		0.37	0.29		0.32	0.33	
B	0.41	0.48		0.42	0.44		1.00	1.23		0.79	0.71	
F	0.20	0.47		0.31	0.25		0.25	0.20		0.12	0.16	
E	0.15	0.10		0.16	0.12		ND	ND		0.20	0.16	
H	0.12	0.20		0.12	0.22		ND	ND		0.16	0.24	
Mean	0.34	0.32		0.39	0.30		0.54	0.58		0.32	0.32	
Median	0.20	0.34		0.31	0.25		0.37	0.29		0.20	0.24	
p value *	p=0.89			p=0.41			p=0.73			p=0.97		
										p=0.41		

ND denotes not detected (table (<0.1%))

* 2-tailed Wilcoxon Matched Pairs Test

Frequencies of WT1-specific cells after stimulation with WT1-pepmix in untreated and alloanergized donor T cells

Table 2

Donor	Percentage of CD8 ⁺ cells			Percentage of CD4 ⁺ cells		
	IFN- γ ⁺		CD107a ⁺	IFN- γ ⁺		IFN- γ ⁺
	Untreated	Alloanergized	Untreated	Alloanergized	Untreated	Alloanergized
AD	0.250	0.100	0.390	0.330	ND	ND
BC	0.230	0.190	0.710	0.810	0.370	0.330
AG	0.200	ND	0.340	0.370	0.260	0.290
BD	0.200	0.180	0.800	0.550	0.220	0.260
BA	ND	ND	ND	ND	0.210	0.380
AE	0.160	0.200	ND	ND	0.100	ND
Mean	0.21	0.13	0.45	0.41	0.23	0.25
Median	0.20	0.18	0.39	0.37	0.22	0.29
p value *	p=0.19		p=0.88		p=0.82	

ND denotes not detectable (<0.1%)
* 2-tailed Wilcoxon Matched Pairs Test